LIMITED POSTNATAL FEEDING REDUCES THE PPARγ-SETD8-H4K20ME^{1}-WNT PATHWAY IN THE LUNG OF CHRONICALLY VENTILATED PRETERM LAMBS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Preterm neonates supported by invasive intermittent mechanical ventilation (IMV) often experience poor postnatal growth characterized by feeding intolerance and failure to thrive. One consequence of poor postnatal nutrition is reduced substrate availability such as essential fatty acids, and reduced alveolar formation, all resulting in poor lung outcomes and risk for chronic lung disease.

Our group showed that preterm lambs supported by IMV have feeding intolerance, poor growth, and impaired alveolar formation. In contrast, preterm lambs supported by high-frequency nasal ventilation (HFNV) feed and grow, and have normal alveolar formation. When preterm lambs supported by HFNV have feeding volumes restricted, they experience poor postnatal growth, and have arrested alveolar formation. One pathway involved in alveolar formation is the essential fatty acid docosahexanoic acid (DHA) activated PPARγ-Setd8-H4K20me^{1}-Wnt pathway.

We hypothesized that restricted feeding of preterm lambs supported by HFNV reduces available serum DHA, as well as components of the PPARγ-Setd8-H4K20me^{1}-Wnt pathway.

To test this hypothesis, we used the preterm lamb model of chronic lung disease. Two groups of preterm lambs were supported by noninvasive HFNV for 21 days. One group (restricted) had feedings restricted to 75% (mL/kg/day) of that received by the control group (matched for feeding tolerated during IMV).

The other group (control) received feedings as tolerated, progressing to *ad libitum*. Both groups were fed ewe's colostrum for three days, proceeding to mature ewe milk. We measured plasma and lung DHA, as well as components of the PPARγ-Setd8-H4K20me^1-Wnt pathway.

Restricted feeding of preterm lambs reduced serum and lung DHA levels relative to control. Restricted feeding of preterm lambs also significantly decreased lung PPARγ, Setd8, and Wnt signaling components β-catenin, and Wnt11 compared to control. Restricted feeding also reduced Wnt output gene MMP9 mRNA transcript level compared to control.

In conclusion, restricted feeding of preterm lambs managed by HFNV reduces serum DHA and components of the PPARγ-Setd8-H4K20me^1-Wnt pathway in the lung. We speculate that restoration of serum DHA levels in preterm lambs managed by restricted feeding during HFNV will restore PPARγ-Setd8-H4K20me^1-Wnt pathway activation and alveolar formation.

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INTRODUCTION

Preterm neonates often require respiratory support with invasive mechanical ventilation (IMV) after birth, increasing the risk for the development of bronchopulmonary dysplasia (BPD), the chronic lung disease of infancy. BPD is characterized by alveolar simplification and decreased respiratory function (1-3). Even with advances in management of preterm birth, BPD remains a significant problem with 5,000 to 10,000 new cases each year in the United States.

Development of BPD is multifactorial, with mode of ventilation and feeding intolerance as known contributors. BPD often develops following oxygen supplementation via IMV. However, decreased incidences of BPD are seen with noninvasive, nasal continuous positive airway pressure (nCPAP) type ventilation (2, 4, 5). Preterm infants managed on IMV commonly experience poor postnatal growth and restricted nutrition (6, 7).

Clinical studies of premature human infants show that poor fetal growth is a predictor of chronic lung disease (6, 8-10). Common clinical management such as the slow initiation of enteral feedings and parenteral nutrition contribute to poor growth in the preterm infant (5, 11, 12). Improper development of the gut, secondary to prematurity, also contributes to feeding intolerance and restricted nutrition to the neonate. All of these issues contribute to poor postnatal growth and inadequate nutrition, factors which place the preterm infant at greater risk for developing BPD (5).

Animal models have confirmed the role of restricted nutrition and the development of BPD (2, 13). The large animal, clinically relevant, preterm lamb model of chronic lung disease provides an opportunity to test the role of limited nutrition to the neonate (2). Using the preterm lamb model of chronic lung disease, historical studies from our lab showed differences in lung outcomes and feeding tolerance between IMV and noninvasive, high-frequency nasal ventilation (HFNV), a type of ventilation similar to nCPAP ventilation. Preterm lambs managed by IMV had feeding intolerance, poor growth, and poor lung outcomes compared to preterm lambs managed by HFNV (14). Lung histology in lambs managed by IMV showed alveolar simplification, characterized by thick mesenchymal walls, short, thick, secondary septa, and decreased apoptosis (Figure 1) (14). Lung histology in lambs managed by HFNV display normal alveolar formation. The results of this study indicate an interplay between mode of ventilation and nutrition.

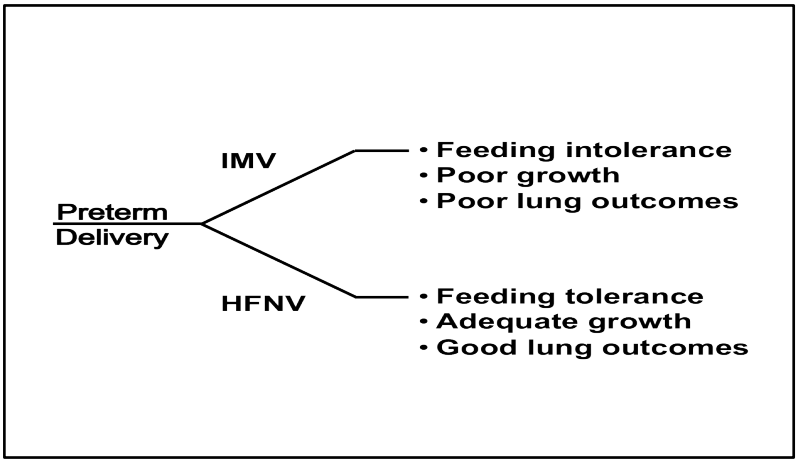

Figure 1 IMV vs. HFNV ventilation type with lung and growth outcomes

To evaluate the role of nutrition in lung outcomes of preterm lambs, our lab conducted a second study with two groups of preterm lambs both managed by HFNV. One group of preterm lambs had feeding volumes restricted to match the feeding intolerance exhibited by the historical IMV managed lambs (restricted feeding group); this included milk feedings of ~75% of the control group milk volume. The control group consumed milk as tolerated (Figure 2). The results showed the restricted feeding group consumed significantly less and grew less than the control group.

The lung histology of the control group showed normal lung development. In contrast, the restricted feeding group had thick mesenchymal walls, and short secondary septa, indicative of poor alveolar formation (Figure 3).

While nutrition appears to affect lung alveolar formation, the mechanism remains unknown. One major molecular pathway involved in alveolar formation is the PPARγ-Setd8-H4K20me^{1}-Wnt signaling pathway (15, 16).

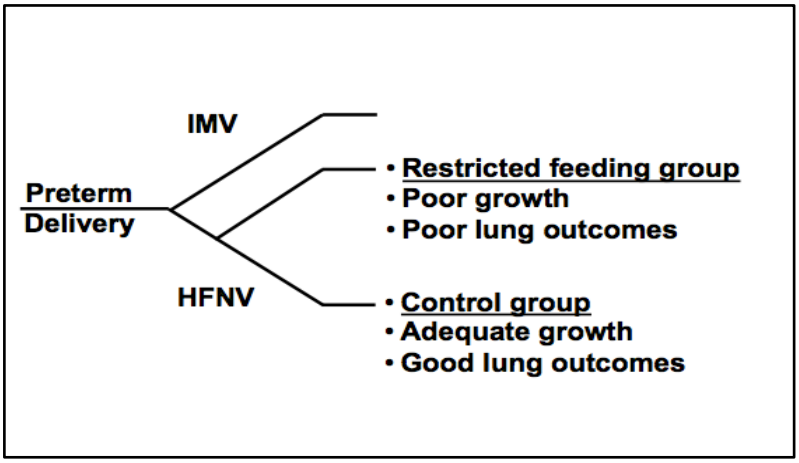

Figure 2 HFNV restricted feeding and control group with lung and growth outcomes

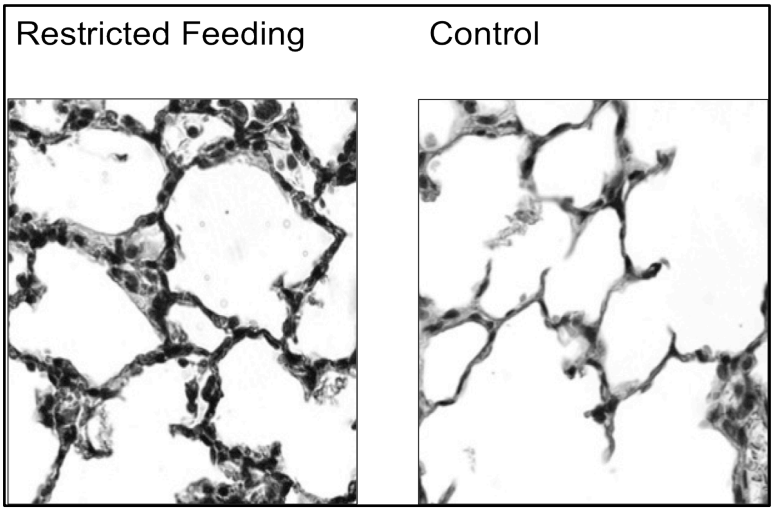

Figure 3 Histopathology for HFNV restricted feeding and control

At the initiation of the pathway is peroxisome proliferator receptor gamma (PPARγ). PPARγ is a member of the nuclear receptor family of transcription factors. Ligand activation of PPARγ results in the transcription of the chromatin-modifying enzyme Setd8 (17). Setd8 is the primary mechanism for the monomethylation of histone 4 ($H4K20me^1$), which is an epigenetic modification (17, 18). In order for Wnt signaling to occur, the $H4K20me^1$ epigenetic mark must be in place (18). Briefly, in Wnt signaling, Wnt components such as Wnt11 and Wnt7a are activated, which allows for the accumulation of β-catenin in the cytoplasm. β-catenin translocates to the nucleus, and acts as a transcription factor for gene enhancers in the TCF/LEF family. LEF1 activation results in the transcription of genes integral in lung structural development, such as matrix metallopeptidase 7 and 9 (MMP7 and MMP9) (19). The final transcription of Wnt signaling output genes such as MMP9 promotes proper lung and alveolar formation (Figure 4).

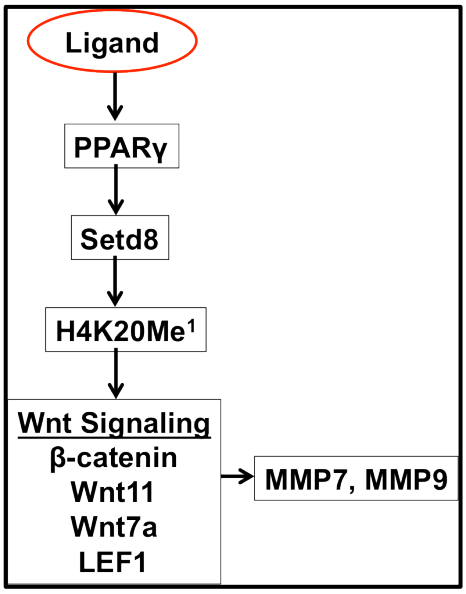

Figure 4 The PPARγ-Setd8-H4K20me^1-Wnt signaling pathway

One ligand with a high binding affinity for PPARγ is the essential omega-3 polyunsaturated fatty acid docosahexanoic acid (DHA) (16, 20-22). DHA is of special interest nutritionally for preterm infants, as it must be obtained via the placenta during the final trimester, a period of gestation often missed due to preterm birth (12, 23, 24). DHA is also physiologically relevant in chronic lung disease. Specifically, infants who develop lung disease have decreased serum levels of DHA (12, 25). The effect of restricted nutrition on DHA status and the PPARγ pathway activation in the lung of preterm lambs is unknown.

We hypothesized that restricted feeding in preterm lambs managed by HFNV will lead to decreased serum DHA. We further hypothesized that restricted feeding will decrease flux through the PPARγ-Setd8-H4K20me^1-Wnt signaling pathway, contributing to the previously observed poor lung outcomes.

METHODS

We used a well-established, IACUC (#11-11002) approved, preterm lamb model in accordance with guidelines of the University of Utah Animal Care Committee. Briefly, pregnant ewes were given antenatal steroids 36 hours before delivery. Lambs were surgically delivered preterm at about 131 days gestation, where term is 150 days. Immediately following birth, lambs were intubated and treated with surfactant and caffeine citrate. All lambs were intubated with intermittent mandatory ventilation for three hours to ensure proper, initial oxygenation. After three hours, the lambs were extubated and placed on high-frequency nasal ventilation for the remainder of the study. Once on HFNV, lambs were placed in one of two groups: either the restricted feeding group or control group.

All lambs were given colostrum for the first three days of life, and mature ewe milk for the remainder of the study with Sav-A-Lamb® milk replacer. Feedings were initially given via orogastric tube as lambs were weaned from IMV to HFNV. Bottle feedings were given when tolerated, and continued through the completion of the study. Feedings were provided and recorded every two hours.

Restricted feeding lambs had their volumes reduced to match volumes tolerated by historical IMV lambs in mL/kg/day, whereas control lambs were fed *ad libitum.* Preterm lambs in both groups were maintained with respiratory gas parameters within physiological ranges. Both groups of preterm lambs were

managed by HFNV for 21 days. After 21 days, lambs were terminated, with serum and lungs collected for analysis.

Mass Spectrometry

Plasma and lung fatty acids were quantified using mass spectrometry by Dr. Camilia Martin's laboratory in Harvard, Massachusetts. Data are expressed in %mol of total fatty acids.

Western Blotting

Western blots were used to measure protein abundance of PPARγ, Setd8, and β-catenin with techniques previously described by our lab (16, 26). Briefly, total protein was isolated by homogenizing lung tissue in RIPA buffer and protease inhibitor (PI) cocktail (Roche-Complete Mini). Samples were centrifuged, and stored at – 80°C until use. Protein abundance was quantified using the Pierce BCA protein assay kit (ThermoScientific) and was stored at -80°C until use.

Total (30 ug) protein was loaded and separated on Nu-PAGE 10% Bis-Tris Midi Gels (Novex by Life Technologies). Proteins were transferred to PVDF membranes (Milli-pore). The antibodies used were PPARγ (DB134, Delta Biolabs), Setd8 (GTX119440, GeneTex), and β-catenin (Cell Signaling 9582). Antibodies were detected with Western Lightning enhanced chemiluminescence and quantified using an Image Station 2000R (Eastman Kodak).

Quantitative Real-time RT-PCR

Quantitative real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) was used to measure mRNA levels of Wnt signaling components. An initial RNA-seq performed by our lab revealed differences between Wnt11 and MMP9 gene expression between restricted feeding and control lambs; as a result, we chose to quantify expression of these genes in this study.

RNA was extracted from lung tissue samples using an RNeasy Mini Kit (Qiagen). cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

The Life Technologies Assay-on demand primer/probe sets used for Wnt11 were (Bt03255381_m1). Custom designed primer and probe sequences were used for MMP9 (see Appendix). GAPDH was used as an internal control. Data and RT-PCR amplification were analyzed by QuantiStudio 12K Flex Real Time PCR system, with mRNA levels determined consistent with the comparative CT method (27).

Statistical Analysis

Data are presented as mean ± standard deviation. Measurements of plasma DHA used an n=3 for each group. At the time of the study, only n=1 sample was available for lung DHA measurement, and therefore, statistical analysis was not applied. For PPARγ, Setd8, β-catenin, Wnt11, and MMP9, means were compared using nonparametric Mann-Whitney U test; n=3-4 samples per group. Statistical significance was accepted at $p \leq 0.05$.

RESULTS

Effects of Restricted Feeding on Plasma Docosahexanoic Acid

Preliminary plasma and lung DHA abundance was quantified in the restricted feeding and control groups of preterm lambs managed by HFNV. For plasma DHA, lambs in the restricted feeding group had a nonsignificant reduction in plasma DHA compared to control lambs (p=0.06, n=3) (Figure 5). For lung DHA levels, the restricted feeding group also had reduced DHA in lung compared to control lambs (Figure 6). However, no statistical analysis could be applied due to an n=1 in the restricted feeding group.

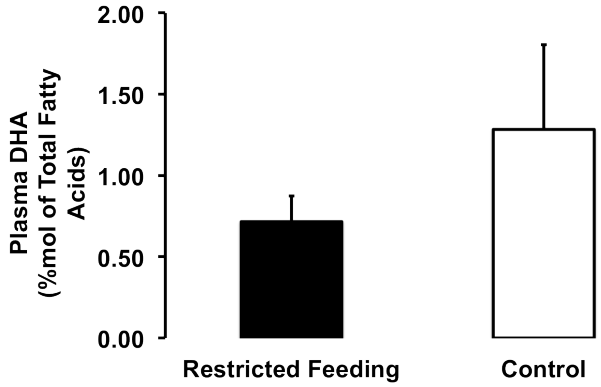

Figure 5 Plasma DHA %mol relative to total fatty acids

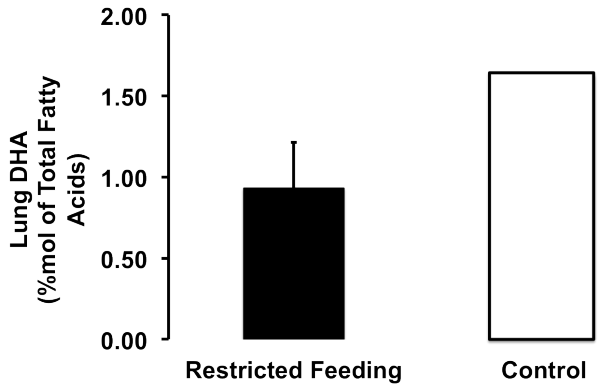

Figure 6 Lung DHA %mol relative to total fatty acids

The PPARγ-Setd8-H4K20me[1]-Wnt Signaling Pathway

Components of the PPARγ-Setd8-H4K20me[1]-Wnt signaling pathway were measured. Protein abundance of PPARγ, Setd8, and β-catenin was quantified from lung tissue. The restricted feeding group had significantly less PPARγ protein abundance in the lung compared to the control group ($p<0.05$) (Figure 7). Protein abundance of Setd8 was significantly reduced in the restricted feeding group compared to controls ($p<0.05$) (Figure 8). Wnt signaling component β-catenin was also significantly reduced in the restricted feeding group compared to control ($p<0.05$) (Figure 9). For Wnt signaling component Wnt11, mRNA were quantified and compared between groups. The restricted feeding group had significantly less Wnt11 mRNA abundance ($p=0.03$) compared to the control (Figure 10).

Lung mRNA levels of Wnt signaling output gene MMP9 were also measured. MMP9 mRNA in the restricted feeding group was trending towards reduction compared to control; however, it was not statistically significant ($p=0.22$) (Figure 11).

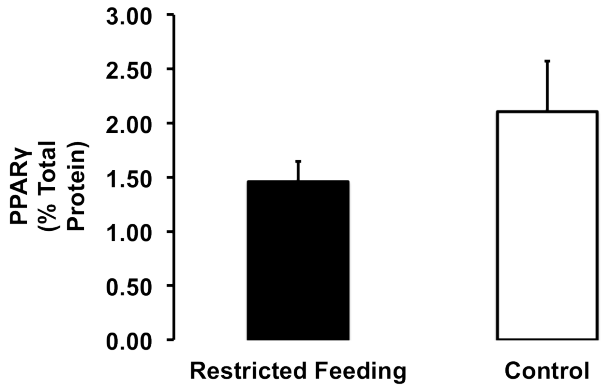

Figure 7 PPARγ protein abundance

*Denotes statistical significance relative to control, $p \leq 0.05$

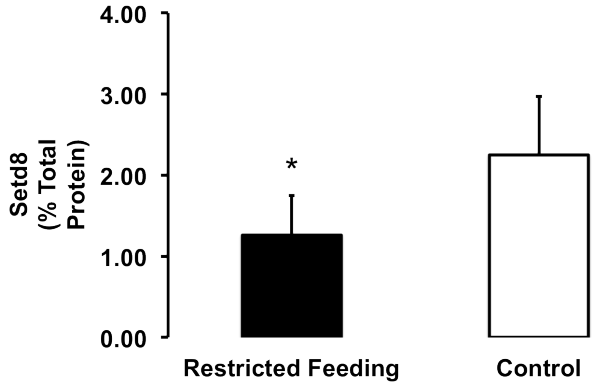

Figure 8 Setd8 protein abundance

*Denotes statistical significance relative to control, $p \leq 0.05$

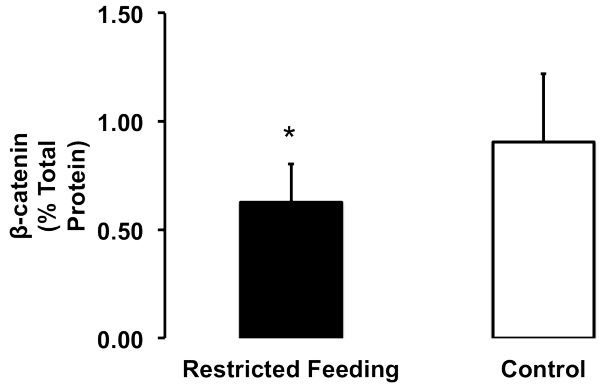

Figure 9 β-catenin protein abundance

*Denotes statistical significance relative to control, $p \leq 0.05$

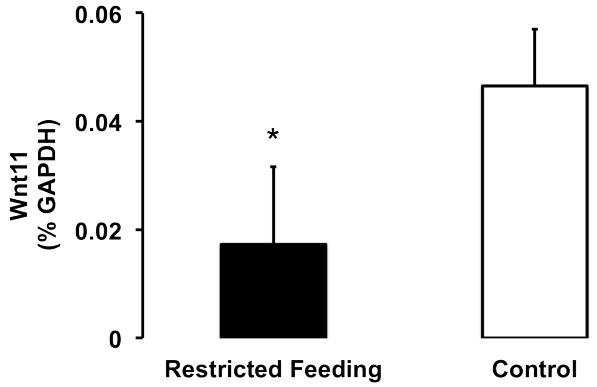

Figure 10 Wnt11 mRNA abundance

*Denotes statistical significance relative to control, $p \leq 0.05$

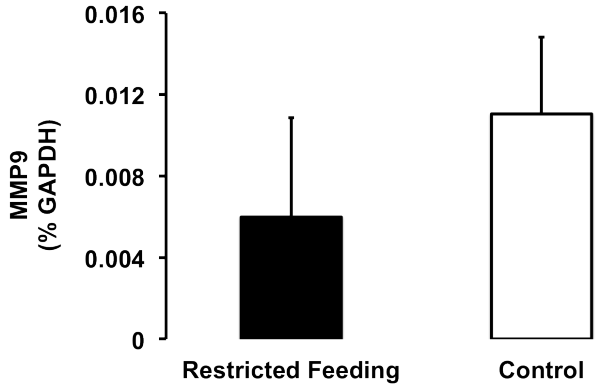

Figure 11 MMP9 mRNA abundance

DISCUSSION

The results of our study show the impact of nutrition on a molecular mechanism of lung development. An overall restriction of feeding of preterm lambs led to reduced flux through components of the PPARγ-Setd8-H4K20me^{1}-Wnt signaling pathway. Despite limited samples, our preliminary data imply that plasma and lung DHA levels may also be reduced by restricted feeding in preterm lambs managed by HFNV. Together, our results suggest a novel mechanism for alveolar simplification in restricted feeding of preterm lambs managed by HFNV.

DHA is an important essential fatty acid, with disturbances implicated in both preterm birth and altered lung development. Our results suggest that restricted feeding leads to decreased plasma DHA and possibly reduced lung DHA. These findings support research that restricted feeding secondary to prematurity is linked to marked differences in available essential fatty acids (12, 25).

Research on DHA and lung outcomes shows that DHA supplementation is linked to improved lung outcomes (28). Enteral formulas for preterm infants are supplemented with DHA, but not at the level required to replete low stores of DHA after preterm birth (12). Normal breast milk also does not contain adequate DHA to replete stores for the preterm infant. However, maternal supplementation of DHA in breast-fed premature infants improved lung outcomes (12).

Parenteral nutrition, while not explored in our study, is linked specifically to the development of chronic lung disease likely due to its lipid emulsion lacking in DHA (12). Clinical studies with preterm infants receiving parenteral nutrition using lipid emulsions containing fish oil (DHA) showed less incidence of chronic lung disease (29). Our findings of reduced plasma DHA support the notion that DHA may be a key component in arrested lung development in preterm infants.

As plasma DHA is a ligand and activator of PPARγ, limited DHA availability is likely to be linked to decreased activation of the PPARγ-Setd8-H4K20me^1-Wnt signaling pathway in the lung (21, 22). While unable to make definitive conclusions based on our sample size, our data strongly suggest that decreased DHA in conditions of restricted feeding may contribute to less activation of PPARγ in conditions of restricted feeding.

Limited activity of PPARγ results in decreased activity of its downstream targets such as the enzyme Setd8 (17). We found that PPARγ and Setd8 protein abundance was reduced in lung tissue under conditions of restricted feeding, suggesting reduced PPARγ protein leads to reduced Setd8 protein. Decreased PPARγ and Setd8 protein abundance in conditions of restricted feeding support previous research from our lab in the rat, and support the role of limited DHA in impaired alveolarization (16).

Decreased abundance of Setd8 may hold implications for the proper activation of Wnt signaling. The major role of Setd8 is to place the epigenetic mark (H4K20me^1) on histone 4. With reduced Setd8, we expect to see differences in genome-wide methylation of histone 4. The epigenetic monomethylation of histone 4 (H4K20me^1) mediates the activation of Wnt

signaling (18).

Wnt signaling is known to be integral and active during lung development (15). We found that Wnt11 and β-catenin were decreased in conditions of restricted feeding, suggesting impaired Wnt signaling. A decrease in components of Wnt signaling in preterm lambs with restricted feeding is consistent with our previous observations of altered alveolar formation, as Wnt signaling output genes are linked to lung development (15). For example, Wnt11 is critical for lung mesenchymal formation (30). Wnt signaling components, such as Wnt11, lead to accumulation of β-catenin in the cytoplasm (19). β-catenin is integral in promoting Wnt signaling (15).

Proper accumulation of β-catenin allows initiation of other pathways leading to transcription of Wnt signaling output genes (15). The output genes of Wnt signaling such as the matrix metalloproteinases (MMPs) are involved in structural formation of lung tissue (15, 31). We found that restricting feeding led to decreased mRNA abundance of MMP9. Decreased MMP9 has been shown to be involved with impaired alveolarization (32, 33). Therefore, reduced MMP9 may be one mechanism by which conditions of restricted feeding contribute to previously observed structural differences in alveolarization.

Our study is not without limitations. Each lamb in our model requires constant 24-hour supervision and management, driving the cost of the experiment high, and the sample size low. Comorbidities of preterm birth are common in the preterm lamb model, similar to preterm human infants, making consistent sample collection difficult. Our study demonstrated the association between available DHA and the PPARγ-Setd8-H4K20me^{1}-Wnt signaling

pathway. However, we did not demonstrate a cause-and-effect relationship. Future studies isolating the connection between available DHA and its role as a ligand in PPARγ activation are warranted. Currently, we are conducting studies using DHA supplementation in conditions of restricted feeding with the goal of normalizing plasma DHA levels to those of term lambs, and correcting arrested alveolarization. We are also conducting studies to determine the effect of PPARγ antagonists in conditions of *ad libitum* feeding and the downstream effects on the PPARγ-Setd8-H4K20me^1-Wnt signaling pathway.

A further limitation of our study is that we did not measure levels of H4K20me^1 in the lung of our study lambs. We are currently conducting ChiP-sequencing in order to determine genome-wide differences in methylation of histone 4 (H4K20me^1). Results of the ChiP-sequencing will demonstrate the effects of reduced Setd8 on overall H4K20me^1, and may indicate which regions of the genome may contribute to lung development.

CONCLUSION

In conclusion, restricted feeding of preterm lambs reduces available serum and lung DHA, and may be linked to decreased activation of PPARγ. Restricted feeding of preterm lambs also reduced flux through components of the PPARγ-Setd8-H4K20me^{1}-Wnt signaling pathway. Our results support an important role of nutrition and one molecular pathway integral in lung development.

APPENDIX

Table 1 Primer/Probe Sequences for MMP9 and GAPDH

GENE	**SEQUENCE**
MMP9	*Forward:* GTGGCACCATCACAACATCAC
	Reverse: CGCGCGGCAGGTCTT
	Probe: TACTGGATCCAAAATTACTC
GAPDH	*Forward:* CAAGATGGTGAAGGTCGGTGT
	Reverse: CAAGAGAAGGCAGCCCTGG
	Probe: GCGTCCGATACGGCCAAATCCG

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